BIKEN'S JOURNAL 4, 41-49 (1961)

Studies on Poly-β-Hydroxybutyrate in Bacterial Spores**
II Localization of Poly-β-Hydroxybutyrate in Relation to the Morphological Structure of Mature Spores of Bacillus cereus

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(Received for publication, February 4, 1961)

SUMMARY

Electronmicroscopic studies were made on mature spores of *Bacillus cereus* No. 2 during treatmen t with organic solvents for the removal of poly-β-hydroxybutyrate and of exosporia obtained by mechanical means. The exosporium could be stripped off from an intact mature spore by drastic treatment with acetone and with an ether-ethanol mixture leaving the spore body. When the spore body was exposed to boiling chloroform remarkable morphological changes were seen on its surface and the poly-β-hydroxybutyrate was concomitantly extracted into the solvent. On the other hand, no polymer could be extracted from a purified preparation of exosporia obtained by mechanical means and only a slight morphological changes were seen after treatment with boiling chloroform. These observations suggest that poly-β-hydroxybutyrate in mature spores may be localized not in the exosporium but in the inner coat of the body.

INTRODUCTION

In a previous communication (Yoneda and Kondo, 1959) we described the existence of poly-β-hydroxybutyrate in mature spores and its relation to the acid-fast stainability of the spores. Mature spores contained a considerable amount of the polymer and its removal caused a loss of acid fast stainability of the spores leaving bodies which stained strongly with ordinary basic dyes. Examination of mechanically disintegrated spores showed that poly-β-hydroxybutyrate was localized exclusively in the insoluble components of the sonically disintegrated materials, suggesting an association of this polymer with some surface structure of the spore body.

It has already been recognized by various workers (Chapman *et al.*, 1953; Robinow, 1953; Bradley *et al.*, 1958) that the mature spore of *Bacillus cereus* has

* Aided by a grant from the Ministry of Education, Japan
** An outline of this work was reported at the 12th Meeting of the Japanese Bacteriological Association in the KANSAI district (October 3, 1959)

a well defined exosporium. The problem therefore arises of whether the polymer is localized in the exosporium or in the inner coat of the spore. It could be that the polymer exists as one of the surface layers which cover the spore core.

The aim of this study is to determine with which structure of the spores poly-β-hydroxybutyrate is normally associated. This information will be useful for an understanding of the importance of poly-β-hydroxybutyrate to the spores.

MATERIALS AND METHODS

1. *Organism and cultural methods*

A laboratory strain of *Bacillus cereus* No. 2 from the collection of our Institute was used throughout this work. Mature spores were produced on a broth agar plate by culturing the organism at 35°C for a week. After incubation, the whole culture still containing a few vegetative cells was harvested, washed three times with chilled distilled water by centrifugation and resuspended in M/100 phosphate buffer (pH 7.0). The suspension was further incubated at 35°C with shaking until very few vegetative cells were detectable. This aerated suspension was again washed three times with chilled distilled water by centrifugation at 3,000 rpm for 15 minutes and the packed, washed spore preparation free from vegetative cells, their small fragments and granules, was dried *in vacuo* ready for use.

2. *Preparation of exosporia*

Four grams of dried mature spores mixed with about an equal volume of glass powder (100 mesh, Tyston) was moistened by adding a small amount of distilled water and then ground in a mortar and pestle in the cold for three hours. After grinding it up, the thick paste was mixed with five volumes of distilled water and the mixture was vigorously shaken in a stoppered flask. The glass powder and the aggregated spore mass were removed by centrifugation at low speed (1000 rpm for 10 minutes) and the turbid supernatant was again centrifuged at 4,000 rpm for 10 minutes. The sediment was collected and resuspended in the same volume of 5 per cent Carbowax (polyethylene glycol, M.W. 4,000) solution. The suspension was then centrifuged at 3,000 rpm for 10 minutes and the supernatant, which showed a slight turbidity, was again centrifuged at 4,000 rpm for 10 minutes. This procedure was repeated twice. The final sediment, washed twice in distilled water by centrifugation at 4,000 rpm for 10 minutes, was dried *in vacuo* ready for use.

Table 1 shows the preparation procedure for exosporia and an electronmicrograph of the preparation is shown in figure 8.

3. *Preparation of stripped spores*

One gram of dried mature spores was suspended in about 20 ml of 1 per cent sodium desoxycholate solution and treated by sonic oscillation at 10 KC for 30 minutes in the cold. After this sonic treatment, the suspension was centrifuged at 3,000 rpm for 10 minutes and the sediment was resuspended in 1 per cent sodium desoxycholate solution. This resuspended material was shaken at 60 cycle in a Mickle's disintegrator for two hours without glass beads and the sediment obtained by centrifugation at 3,000 rpm for 10 minutes was successively washed in 5 per cent Carbowax, 2 per cent Tween 80 and distilled water by centrifugation at the same speed. The method of preparation is shown in Table 2 and an electronmicrograph of the preparation is shown in figure 10. It was difficult to remove exosporia from this preparation and, as can be seen from the figure, there are still a number of free exosporia mixed with the stripped spores.

4. *Dry-weight measurements*

The dry weights of the intact spores and of the exosporia were estimated by drying measured volumes to constant weight at 120°C.

5. *Staining methods*

Moeller's staining method (1891) with a slight modification and simple staining with Loeffler's methylene blue were employed for mature spores, stripped spores and exosporia before and after treatment with organic solvents, as described in the following sections.

6. *Extraction of poly-β-hydroxybutyrate*

Poly-β-hydroxybutyrate was extracted by the method described in our previous paper (1959).

7. *Electronmicroscopy*

Photographs were made of air dried material on collodion covered grids, after shadow casting with chromium at an angle of about 45°, with an Hitachi HU-9D-electronmicroscope at 75 KV.

Table 1. Method of preparation of exosporia

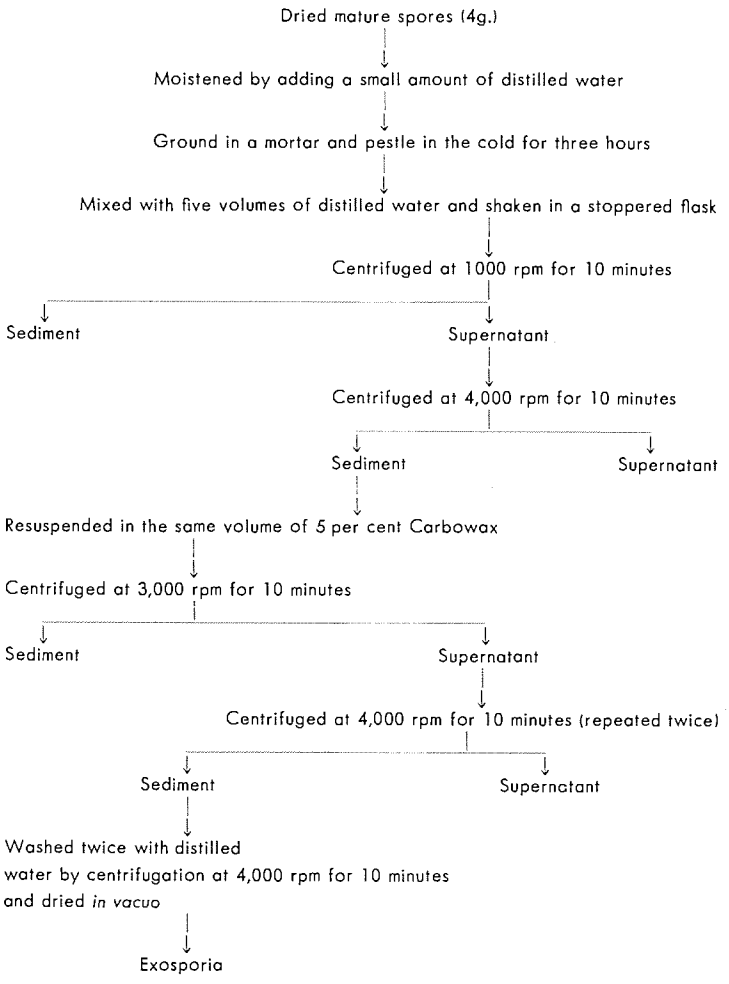
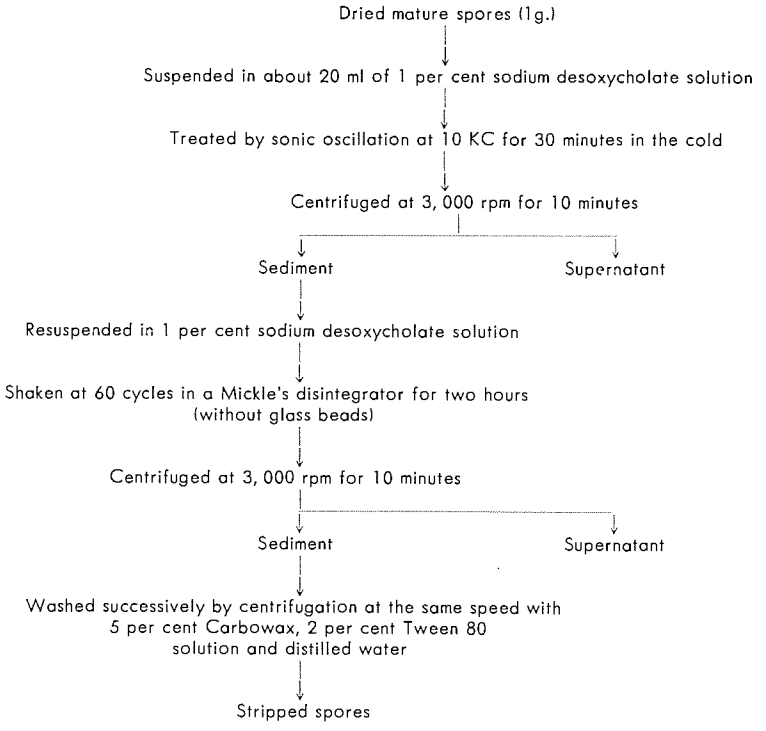


Table 2. Method of preparation of stripped spores



RESULTS

1. *Morphology of mature spores treated with organic solvents*

In our previous paper (1959) it was shown that remarkable changes occured in the staining properties of mature spores of *Bacillus cereus* No. 2 during successive treatment with acetone, an ether-ethanol mixture (1:3, v/v) and chloroform, suggesting that extraction of lipids from th spores may also seriously affect their surface morphology. Therefore, dried mature spores were treated as described in our previous report with these solvents, and the change in their appearance was examined electronmicroscopically after each extraction step. The staining properties of smears taken from the specimens for electronmicroscopy were also examined at each step.

Figures 1-7 show some of the many electronmicroscopic photographs of untreated dried mature spores of *Bacillus cereus* No.2 and of spores taken during successive treatment with acetone, an ether-ethanol mixture and chloroform.

a) *Untreated dried mature spores*

As shown figure 1, untreated spores of the *B. cereus* strain used in this study have

an well defined exosporium protruding considerably over the poles of rather long oval shape spore body. It is of some interest that the electron dense body has an irregular shaped edge, particularly noticeable at the ends of the cell. It is as yet uncertain, however, whether this is an artifact due to drying or the normal structure. These spores are acid fast in stain and stained pinkish with carbol basic fuchsin solution.

b) *Mature spores treated with acetone*

Mature spores were treated with boiling acetone for three hours and the residual spores were subjected to electronmicroscopy and to Moeller's stain. Although they stained very much as untreated spores, they differed markedly from the latter in their appearance in electronmicroscopy and, as is shown in figure 2, some damage and distortion is seen in the exosporium, whereas the spore body appeared to be intact. At this stage of treatment, considerable lipid could be extracted in acetone, evaporation of which left a dark yellow residue. These observations suggest that ether-soluble lipids must be components of the exosporium.

c) *Mature spores successively treated with acetone and an ether-ethanol mixture*

The spores obtained after extraction with acetone were exposed to a boiling ether-ethanol mixture (1:3, v/v) for three hours. Removal of the lipids soluble in this solvent appeared to increase the damage of the exosporium and the distortion became much clearer, resulting in the formation of spores whose exosporium had been stripped off (fig. 3). Spores completely stripped of their exosporium could sometimes be seen at this stage (fig. 4) and the number of these stripped spores increased with the time of further extraction. The staining properties of such spores are of considerable interest. Thus, accompanying the deformation found by electronmicroscopy, they stained more deeply with carbol basic fuchsin than untreated or acetone-treated spores. From figure 4 one can see an outer translucent layer surrounding an electron dense body. It may be that this layer is the spore membrane previously described by Robinow (1951). Whatever it may be, however, it is of interest that, even after this drastic treatment, no deformation was observable in the spore body. This layer at least must be composed of material which is not extracted by these solvents.

It should be noted here that some difficulty was encountered in obtaining good specimens for electronmicroscopy because most spores tend to aggregate or to stick together forming amorphic masses after treatment with these solvents. This was also the case in the chloroform treated preparation described below.

d) *Mature spores successively treated with acetone, an ether-ethanol mixture and chloroform*

The spores, after treatment with acetone and an ether-ethanol mixture were further treated with boiling chloroform for three hours. During this procedure, specimens for electronmicroscopy and staining were taken after 10, 60 and 180

minutes respectively.

Striking changes in the appearance of the spore bodies were observed during this chloroform treatment as shown in figure 5, 6 and 7. Destruction of the spore body appears to be initiated by a deformation of the surface. Thus, after a short contact (ten minutes) with this solvent, the spore body began to be deformed, particularly the surface and the delicate translucent outermost layer could no longer be seen (fig. 5). This layer seemed to dissolve in the solvent. Incidentally a considerable amount of poly-β-hydroxybutyrate was extracted by this treatment, as reported in our previous paper. Deformation of the spore body appeared to develop with continueing extraction and a remarkable destruction of the entire body took place during the treatment (fig. 6 and 7). At this stage a number of small bodies were seen in smears, which stained deeply with ordinary alkaline methylene blue solution indicating that they were no longer acid fast. On the other hand, the material extracted by the chloroform, which we had previously shown to be poly-β-hydroxybutyrate (1959), showed a typical acid-fastness in the stain.

2. *Effect of chloroform-treatment of a purified preparation of the exosporia*

From the results described in the preceding section, it may be supposed that poly-β-hydroxybutyrate is associated not with the exosporium but with the inner coat of mature spores of *Bacillus cereus* No.2. This hypothesis can be validified by demonstrating the absence of this polymer from the exosporium.

Approximately 100 milligrams of a purified preparation of exosporium obtained by the method described in the preceding section was exposed to boiling chloroform for three hours. Assay of the polymer content of the solute was then made and the residual exosporia were examined with a light and electronmicroscope.

Figure 8 illustrates one of the electronmicrographs of our preparation of the exosporium. Careful examination by electronmicroscopy showed only a very few stripped spore bodies. These untreated exosporia did not stain with alkaline methylene blue or carbol basic fuchsin in Moeller's stain. Figure 9 shows an enlargement of a photograph of one of the exosporia treated with chloroform as described above.

It may be seen that no appreciable change takes place in the appearance even after this drastic treatment.

Only traces of poly-β-hydroxybutyrate were extracted from 100 milligrams of a purified preparation of exosporium. This was also the case when the same preparation was successively treated with acetone, an ether-ethanol mixture and chloroform. The results indicate the absence of poly-β-hydroxybutyrate from the exosporium of *Bacillus cereus* No.2. Traces of the polymer found in the solute may be accounted for by polymer in the few stripped spores still in the preparation.

3. *Staining properties of stripped spores of Bacillus cereus No.2 obtained by mechanical means.*

As mentioned in the preceding section, when mature spores were drastically treated in succession with acetone and an ether-ethanol mixture, the exosporia appeared to be stripped off and the spores stained deeply with carbol basic fuchsin in Moeller's stain. Assuming that poly-β-hydroxybutyrate which causes the acid-fast stainability of the spores, is really localized in some surface structure of the spore body, this may also be the case with spores whose exosporia were stripped off by mechanical means.

Stripped spores were thus made from mature spores of *Bacillus cereus* No.2 by the method described in the section on MATERIALS AND METHODS, and were examined by light and electronmicroscopy.

Figure 10 shows an electronmicrograph of our preparation. It can be seen that most of the spores are stripped or partially stripped of their exosporia which still remain in this specimen. Attempts have so far failed to remove the exosporia and debris completely from the preparation. The staining properties of these stripped spores with Moeller's stain were as expected. Thus, they stained deeply with carbol basic fuchsin, showing a typical acid fast stainability. It is interesting to note here, however, that, with continued grinding with glass powder, some of the spores became stainable with methylene blue and these non-acid fast spores increased with the time of grinding.

DISCUSSION

The existence in mature spores of *Bacillus cereus* No. 2 of poly-β-hydroxybutyrate with acid-fast stainability was reported in our previous paper (1959). It was also found that the polymer was localized exclusively in the insoluble fraction of mechanically disintegrated spores. The results described in this paper confirm this observation and further, evidence is presented that the polymer is invariably associated with some of the spore coats but certainly not with the exosporium surrounding the spore body. This evidence may be summarized as follows: a) The initial deformation of the surface structure of the spore body occurs at the same time as extraction of the poly-β-hydroxybutyrate with chloroform. b) The polymer is absent from isolated exosporia. c) The exosporia are not acid fast when stained. d) The acid-fast stainability of naked spore bodies obtained by stripping off the exosporia either by mechanical treatment or by treatment with acetone and an ether-ethanol extraction treatment is retained. e) The acid-fast stainability of the spore bodies is lost after prolonged mechanical treatment or chloroform extraction.

In 1956, Chapman reported that spores of *Bacillus cereus* were characterized by the three spore coats surrounding the spore core. Our preliminary observations on ultra-thin sections suggest that this may also be the case in mature spores of

Bacillus cereus No. 2 used in this study. The question therefore arises as to which spore coat is associated with the poly-β-hydroxybutyrate. As described in the RESULTS section, the naked spore body is always found to have an outermost translucent layer and the first sign of deformation in the spore body by the chloroform-treatment for extracting poly-β-hydroxybutyrate is seen in this layer. This layer appears to dissolve in the solvent. Indeed, at this stage of extraction, a considerable amount of the polymer is removed from the spore body. These findings strongly suggest that the polymer may be a major constituent of this outermost layer. It may even be that this layer is a thin membrane of poly-β-hydroxybutyrate itself. However, this is only a speculation and the true answer must await further morphological studies using ultra-thin section techniques.

A combination of grinding with glass powder and differential centrifugations in 5 per cent Carbowax solution yielded an effective and relatively simple method of isolating and purifying exosporia. Since only a very few spore bodies were detectable in the final preparation, it is good material for studying the chemical composition, the enzymatic activities (Berger *et al.*, 1960) and further, immunochemical properties of the exosporium. The chemical composition of the spore membrane of a strain of *Bacillus cereus* was reported previously by Yoshida *et al.* (1957). However, their preparation of the spore membrane appeared to be comprised by both exosporia and spore coats. Incidentally great difficulty was always encountered in purifying naked spore bodies. As described in a previous section, all attempts to completely eliminate exosporia and debris from the spore body preparation failed. There is not very much contamination but still enough to make chemical analyses of the spore coats difficult. Adequate techniques must be devized to resolve this problem.

Our preliminary experiments have shown that the germinating ability of mature spores during treatment with the organic solvents used in this study were largely retained until chloroform treatment. This result is somewhat in line with the findings of Berger and Marr (1960). The true physiological functions of poly-β-hydroxybutyrate in the spore body are yet unknown. However, considering the finding of Berger *et al.* (1960), that naked spore bodies stripped of their exosporia are still viable and heat resistant, and our finding that the poly-β-hydroxybutyrate is localized in some surface coat of the spore body, the polymer may function in some way in protecting the spore core from unphysiological conditions of the environment.

ACKNOWLEDGEMENT

The authors are grateful to Dr. T. Fujino for his interests in this work.

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EXPLANATION OF PHOTOGRAPHS

Fig. 1 : Intact mature spore. (It has a well defined exosporium protruding considerably over the poles of rather long oval shape spore body) *Magnification*, ×16,000

Fig. 2 : Acetone treated spore. (There is some damage and distortion of the exosporium, whereas the spore body appears to be intact) *Magnification*, ×24,000

Fig. 3 : Acetone and ether-ethanol treated spore. (There is clearer damage and distortion of the exosporium than in Fig. 1) *Magnification*, ×20,000

Fig. 4 : Acetone and ether-ethanol treated spore. (Spore completely stripped of their exosporium is sometimes seen at this stage and the number of these stripped spores increased with the time of further treatment) *Magnification*, ×20,000

Fig. 5 : Acetone, ether-ethanol and chloroform treated spore. (After a short contact with chloroform, 10 min., the spore body began to be deformed) *Magnification*, ×20,000

Fig. 6 : Acetone, ether-ethanol and chloroform treated spore. (Deformation of the spore body appeared to increase with continuation of the chloroform treatment, 60 min..) *Magnification*, ×20,000

Fig. 7 : Acetone, ether-ethanol and chloroform treated spore. (Remarkable destruction of the entire body took place during the 120 min. treatment; A number of small bodies staining deeply with ordinary alkaline methylene blue solution were found in smears) *Magnification*, ×20,000

Fig. 8 : Untreated exosporia. *Magnification*, ×6,000

Fig. 9 : Chloroform treated exosporium. (No appreciable change takes place in its appearance even after drastic treatment) *Magnification*, ×15,000

Fig.10: Stripped spores obtained by mechanical means. *Magnification*, ×6,000

Fig. 1 Intact mature spore.

Fig. 2 Acetone treated spore.

Fig. 3 Acetone and ether-ethanol treated spore.

Fig. 4 Acetone and ether-ethanol treated spore.

Fig. 5 Acetone, ether-ethanol and chloroform treated spore. (10 min)

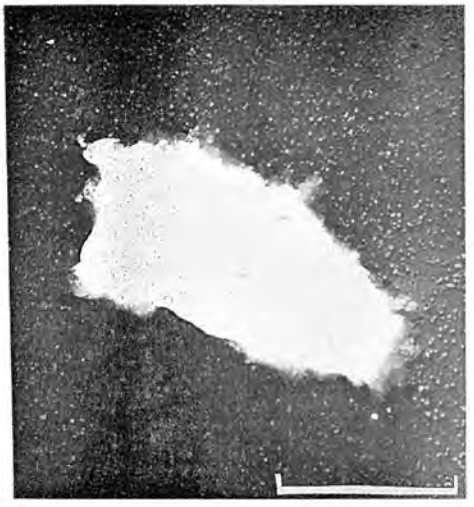

Fig. 6 Acetone, ether-ethanol and chloroform treated spore. (60 min)

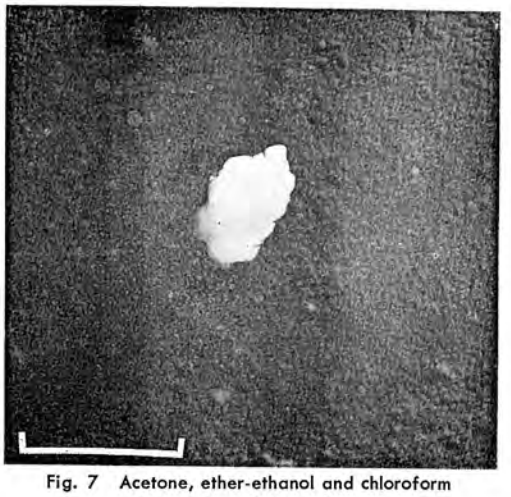

Fig. 7 Acetone, ether-ethanol and chloroform treated spore. (120 min)

Fig. 8 Untreated exosporia.

Fig. 9 Chloroform treated exosporium.

Fig. 10 Stripped spores obtained by mechanical means.